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NITROGEN EXCRETION IN SOME MARINE TELEOSTS[1]

J. D. WOOD

Abstract

The nitrogen-containing compounds excreted by the sculpin, starry flounder, and blue sea-perch were investigated. Approximately four-fifths of the nitrogenous material was excreted via the gills. Ammonia and urea were the main components of the excreta in all three species. The amount excreted in 24 hours varied greatly between fish of any one species, but the percentage composition was reasonably constant. A greater variation in the composition occurred between species. Trimethylamine oxide was present in only small amounts in the total excreta. It was concluded from this that the compound was not an important end product of protein metabolism.

Introduction

The nitrogenous material excreted by the animal body is of interest because it reveals the end products of protein metabolism within the animal. In the fish kingdom the excreta of marine teleosts have been studied extensively and particular attention has been paid to trimethylamine oxide (TMO). This compound was first isolated from fish by Suwa (1), who obtained it from dogfish muscle. It has since been shown to occur extensively in different species throughout the marine world (2). Grollman (3) reported that 37% of the total non-protein nitrogen in the urine of *Lophius piscatorius* was present as TMO-N. This was confirmed by Grafflin and Gould (4). These workers found that sculpin urine likewise was rich in TMO but that flounder urine contained very little of the compound. Urea-N accounted for approximately 15% of the total N in the urines of sculpin and flounder, but little urea was found in urine of *Lophius piscatorius*. Ammonia was present only in small amounts in the urines of all three species.

The physiological role of TMO has been debated for a long time and a review of the different theories is given by Shewan (2). Among the theories mentioned are, (*a*) that TMO is the non-toxic end product of protein metabolism, (*b*) that TMO plays an important part in osmoregulation, and (*c*) that TMO is of exogenous origin, being obtained from the food of the fish.

Smith (5) found that fresh-water teleosts excreted 80% to 90% of the nitrogenous material via the gills and the remainder by the kidneys. Therefore, the percentage composition of the total excreta could be quite different

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from that of the urine since the composition of the total excreta will be influenced mostly by the material eliminated via the gills. If marine teleosts likewise eliminate most of the nitrogenous compounds via the gills, then TMO may not have the significance that the composition of the urine suggests. Delaunay (6) measured the total excreta of the sole and he found that ammonia was the major nitrogenous constituent. This was a very different composition from that found for the urines previously mentioned. Whether the variations in composition were due to differences between species or between total and urinary excretions was not investigated. Neither did Delaunay determine TMO in the excreta of the sole.

A certain confusion seems to have arisen in textbooks concerning the nitrogen excretion of marine teleosts. Prosser (7) reports the results of Delaunay and of Grafflin and Gould but does not differentiate between urinary excretion and total excretion. Baldwin (8) states that one-third of the total excreta of marine teleosts is in the form of TMO, and refers to H. W. Smith's results. However, the latter employed fresh-water teleosts and he did not determine TMO. No report of similar experiments on marine teleosts by Smith could be found in the literature.

The present experiments were carried out to determine the composition of the excreta from different species of marine teleosts with particular reference to TMO.

Materials and Methods

Fish

The species employed in the experiments were the sculpin, *Leptocottus armatus*; the starry flounder, *Platichthys stellatus*; and the blue sea-perch, *Taeniotoca lateralis*. The fish were caught in the Straits of Georgia, British Columbia, and kept in aquaria until required. During captivity they were fed pieces of lingcod muscle.

Collection of Excreta

The fish specimen under consideration was placed in a tank slightly larger than itself and sufficient sea water was added to cover the fish. This usually required between 1 and 2 liters of water. The water was well aerated and was kept at 12° C by placing the tank in a constant flow of cold water. After 24 hours the fish was removed from the tank and the water acidified with a small amount of HCl and taken down to dryness *in vacuo* at 45° C. The non-protein nitrogenous compounds were largely separated from the inorganic salts with four successive extractions of 80% ethanol. The total volume of ethanol used was one-seventh of the original volume of the sea water. The ethanol extracts were combined, filtered, and taken down to dryess *in vacuo* at 45° C. The residue was redissolved in water to give a final volume of 50 ml.

This extraction procedure yielded 97% recovery when applied to known amounts of ammonia, urea, trimethylamine (TM), TMO, and creatine dissolved in sea water.

Analyses

The total N was estimated using the micro-Kjeldahl method as adapted by Ma and Zuazaga (9).

The ammonia-N, TM-N, and TMO-N were determined by the method of Ronold and Jakobsen (10). In this method the ammonia-N may include some methylamine-N and dimethylamine-N. The presence of the latter two compounds can be detected using the Conway microdiffusion cell. If the outer compartment is buffered at pH 9.5 with borate, then the amines diffuse into the center compartment much more slowly than ammonia. After 5 hours at 20° C, 94% of the ammonia had diffused into the center well compared with 9% of the methylamine and 8% of the dimethylamine. The material from sculpin excreta diffused rapidly, 91% being found in the center well after a period of 5 hours. It was therefore concluded that the ammonia determined in the excreta was only slightly, if at all, contaminated with the amines.

Urea-N was measured by the methods of Fearon (11) and Koritz and Cohen (12), and good agreement was obtained between the two methods. These methods estimate compounds containing a ureide grouping, but of all the compounds giving a positive test only urea gives a characteristic bright yellow color using the method of Fearon. The excreta of the fish gave this characteristic color, but to check the identity of the ureide-containing compound more accurately, the absorption spectra of the colored solutions were measured. Table I shows the wavelength of maximum absorption and the

TABLE I

LIGHT ABSORPTION OF COMPOUNDS CONTAINING A UREIDE GROUPING

	Wavelength of maximum absorption (mμ)	Absorption at 484 mμ / Absorption at 495 mμ
Urea	484	1.34
Methyl urea	492	0.93
Citrulline	496	0.78
Sculpin excreta	484	1.33

ratio of the absorptions at 484 mμ and 495 mμ for different compounds and for sculpin excreta. These results show that the material in the sculpin excreta is mostly, if not entirely, urea. The method of Koritz and Cohen was the one employed during the experiments because of its greater sensitivity. The authors of this method point out that biological material sometimes increased slightly the optical density produced by a given amount of compound. In order to eliminate this possible source of error, urea standards were prepared using the excreta solution as solvent. The optical density due to the added urea was then obtained by difference.

Creatine-N and creatinine-N were estimated by the method of Bonsnes and Taussky (13). Prior to these determinations 2 *N* NaOH was added

drop by drop until no further precipitation of inorganic material occurred. The precipitate was removed by centrifugation, resuspended in 0.5 *N* NaOH, and again centrifuged. The two supernatant fractions were combined and after neutralizing with HCl they were ready for use in the estimations. The method of extraction of the excreta from sea water was found to change a small part of the creatine to creatinine. Therefore the creatine-N and creatinine-N values were combined in the results presented here.

Results

An experiment was carried out similar to that described by Smith (5) to determine the proportions of the nitrogenous material excreted by the gills and by the kidneys, respectively. A sculpin was employed as the experimental fish and it was found that 77% of the total N was excreted via the gills.

The excreta from seven sculpins, two starry flounder, and a blue sea-perch were investigated and the results are shown in Table II. The total amount excreted varied considerably between fish of the same species, but in all cases the major components of the excreta were ammonia and urea. The predominance of these compounds in the excreta was observed in all three species of fish investigated.

TABLE II

NITROGEN EXCRETION IN MARINE TELEOSTS

	Sculpin							Starry flounder		Blue sea-perch
	1	2	3	4	5	6*	7*	1	2	1
Weight, g	165	182	191	391	151	245	304	335	310	360
Total N	8.31	7.45	24.88	24.38	6.04	16.22	18.50	22.46	24.52	10.71
Ammonia-N	4.37	4.66	16.77	17.64	4.02	10.11	10.23	19.37	19.97	5.14
Urea-N	1.87	1.53	6.15	4.22	1.02	3.90	3.59	2.58	2.96	4.08
Trimethylamine-N	0.00	0.10	0.39	0.33	0.11	0.46	0.36	0.25	0.05	0.03
Trimethylamine oxide-N	0.18	0.00	0.33	0.93	0.05	0.00	0.04	0.12	0.00	0.04
Creatine-N + creatinine-N	0.03	—	0.23	0.17	0.03	0.18	0.26	0.06	0.30	0.00

NOTE: All N values in the table are given as mg.

*These fish were newly caught and had not been kept in aquaria prior to collection of excreta.

When these results are converted to percentages of the total N excreted, Table III, it is observed that the ammonia and urea contents of the excreta remained reasonably constant within any one species, although the size of the fish varied considerably. Moreover, the newly caught fish showed the same percentage composition of ammonia and urea as did those which had lived for a considerable length of time in the aquarium. The same constancy of composition, however, did not hold from species to species. The starry flounder had a greater percentage of ammonia and less urea than the sculpin, and the blue sea-perch had less ammonia and more urea.

The average undetermined-N values in the excreta of the sculpin, starry flounder, and blue sea-perch were 13%, 3%, and 13% respectively. The

TABLE III

PERCENTAGE COMPOSITION OF EXCRETA FROM MARINE TELEOSTS

	Sculpin							Starry flounder		Blue sea-perch
	1	2	3	4	5	6	7	1	2	1
Ammonia-N	52.6	62.6	67.4	72.4	66.6	62.3	55.3	86.2	81.4	48.0
Urea-N	22.5	20.5	24.7	17.3	16.9	24.0	19.4	11.5	12.1	38.1
Trimethylamine-N	0.0	1.3	1.6	1.4	1.8	2.8	1.9	1.1	0.2	0.3
Trimethylamine oxide-N	2.2	0.0	1.3	3.8	0.8	0.0	0.2	0.5	0.0	0.4
Creatine-N + creatinine-N	0.4	—	0.9	0.7	0.5	1.1	1.4	0.3	0.1	0.0
Undetermined N	22.3	15.6	4.1	4.4	13.4	9.8	21.8	0.4	6.2	13.2

undetermined N was investigated in the sculpin excreta. Tests were made for uric acid and amino acids but neither was detected.

The effects of bacterial action on the excreta were investigated. A sculpin was placed in a tank of water and after 24 hours the fish was removed and a sample of water analyzed for ammonia and urea. The remaining water was left in the tank for a further 24 hours under the same conditions of temperature and aeration as previously. At the end of this time a sample of the water was taken and the ammonia and urea contents compared with the first sample, Table IV. Little change is observed in the contents of the two samples.

TABLE IV

EFFECT OF BACTERIA ON EXCRETA

	Ammonia-N	Urea-N
Water previous to standing for 24 hours	10.95	2.32
Water after standing for 24 hours	10.62	2.25

NOTE: All figures are mg nitrogen per liter water.

TABLE V

CHANGES IN NITROGENOUS COMPOUNDS IN SEA WATER DUE TO THE PRESENCE OF A SCULPIN

Sample	Ammonia -N	Trimethyl-amine-N	Trimethyl-amine oxide -N	Urea -N	Creatine-N + creatinine-N
Water initially	0.6	6.6	8.0	7.2	8.2
Water after containing sculpin for 24 hours	10.4	6.5	8.2	9.1	8.6
Excreted by sculpin in previous 24 hours	7.7	0.0	0.1	1.7	0.2

NOTE: All figures are mg nitrogen.

The possibility was considered that bacteria on the surface of the fish might act on the excreta. This was investigated by placing a sculpin in a solution of sea water containing known amounts of ammonia, urea, TM, TMO, and creatine. At the end of 24 hours the water was analyzed for these compounds,

and the amounts compared with the original quantities. The results are shown in Table V together with the nitrogenous material excreted by the sculpin during the 24 hours previous to the experiment. The changes in the amounts of the original compounds are consistent with what might be expected when the excretion of the sculpin in 24 hours is taken into consideration.

It appears, therefore, that the excreta of the fish were not attacked by bacteria under the conditions being used for the experiments.

Discussion

Although generalizations about marine teleosts cannot be made on the basis of results from only three species, the results reported here suggest that ammonia and urea are the main nitrogen-containing compounds excreted by marine teleosts. Together these compounds account for 75% to 98% of the total N. Of the two compounds, ammonia is present in the greater quantities.

TMO accounts for approximately one-third of the total N in the urine of some species (3, 4). However, the amount of nitrogenous material excreted in the urine is small compared with that excreted by the gills. It is not too surprising therefore to find that the proportion of TMO in the total excreta is relatively small. The low percentage of the compound in the excreta indicates that the compound is not an important non-toxic end product of protein metabolism in marine teleosts as previously suggested (8, 14). The low and rather varied values are more in accordance with the theory of Norris and Benoit (15) that TMO in marine teleosts is obtained directly from the food, i.e. it is of exogenous origin.

Acknowledgment

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EFFECT OF AMBIENT TEMPERATURE ON THERMAL RESPONSES TO DRUGS[1]

IRVING SHEMANO[2] AND MARK NICKERSON

Abstract

Thermal responses to a variety of drugs have been investigated at various ambient temperatures, using unanesthetized rats, either lightly restrained or paralyzed with tubocurarine. The results indicate that ambient temperature is a major factor determining thermal responses to many drugs. Experiments on lightly restrained rats demonstrated that the *critical ambient temperature*, the temperature above which hyperthermia is evoked and below which hypothermia is produced, is about 30° C (in the thermoneutral range) for Hydergine, ergotamine, LSD-25, and serotonin. The critical ambient temperature for chlorpromazine is approximately 36° C, and that for 2,4-dinitrophenol, 20° C. Reserpine produced a consistent hypothermia at 23° C, but somewhat inconsistent effects at ambient temperatures above this up to 39° C. Complete curarization abolished the hypothermic effects of all the above agents except chlorpromazine. The production of both hyperthermia and hypothermia by most of the drugs studied suggests that they influence temperature-regulating centers of the central nervous system.

Most reports dealing with the effects of drugs on body temperature fail to include information about environmental temperature at the time of the experiment or do not attach significance to it. Failure to recognize this variable has led to considerable confusion, well exemplified by the literature on the ergot alkaloids. Every possible type of body temperature response has been attributed to these agents, including no change (1), hypothermia (2,3), and hyperthermia (4,5). Differences in species and dosage have been suggested as possible explanations of these conflicting results (6). However, these factors cannot be responsible for the variable results obtained by the same dose and in the same species. More recently it has been reported that ergotoxine may evoke any one of these responses, the type depending on the ambient temperature (7). A few other reports also have noted modification of thermal responses to various drugs by changes in ambient temperature (8,9,10,11,12).

The present studies were undertaken to assess the role of ambient temperature in determining thermal responses to a variety of drugs. Six agents with some known action on the central nervous system were investigated. These were chlorpromazine (Thorazine, Largactil), ergotamine, Hydergine, lysergic acid diethylamide (LSD-25), serotonin (5-hydroxytryptamine), and reserpine.[3] 2,4-Dinitrophenol, an agent presumed to have a predominantly peripheral action on body temperature, also was included in the study.

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Contribution from the Department of Pharmacology and Therapeutics, University of Manitoba Faculty of Medicine, Winnipeg, Manitoba. Supported by grants-in-aid from the National Research Council of Canada and the Burroughs Wellcome Co., Tuckahoe, N.Y.
[2]Present address: Pharmacology Section, Smith, Kline and French Laboratories, Philadelphia, Pa.
[3]The authors are indebted to Abbott Laboratories for supplying the serotonin used; to Sandoz Pharmaceuticals for the Hydergine, ergotamine, and LSD-25; to the Ciba Co. for the reserpine; and to Smith, Kline and French Laboratories for the chlorpromazine.

Methods

Unanesthetized female albino rats (150 to 200 g body weight) were placed in individual wire mesh holders in a constant temperature room ($\pm 0.5°$ C) and their colonic temperatures recorded with copper–constantan thermocouples inserted five to six centimeters beyond the anal sphincter. The thermocouples were calibrated before and after each experiment; maximal drift during this period was 0.08° C. After a 2-hour equilibration period, the agent under study was injected subcutaneously and colonic temperatures recorded hourly for an additional 3 hours. Each rat was used as its own control during a separate equal period at the same ambient temperature. The drug-induced effect was calculated for each animal as the difference between the maximal temperature change after a drug and the corresponding temperature in the control experiment. Body temperature just prior to drug injection was taken as the basal temperature. The significance of drug-induced changes was calculated by the method of individual (paired) comparison (13).

In another series of experiments, the thermal effects of the above agents on curarized rats were investigated at a room temperature of $16 \pm 1°$ C. Female albino rats (150 to 200 g) were paralyzed with a minimal curarizing dose of tubocurarine chloride (0.75 mg/kg intramuscularly). Tracheotomy was quickly accomplished under local procaine anesthesia, a tracheal cannula inserted, and artificial respiration started. Complete skeletal muscle flaccidity was maintained throughout the experiment with small supplements of tubocurarine. Colonic temperatures were recorded as described above. After a half-hour equilibration period, the agent under study was injected subcutaneously and the colonic temperature recorded every half-hour for an additional 2 hours. Control experiments were carried out on another group of animals under identical conditions. In the calculation of responses, the body temperature just prior to drug injection was taken as the basal temperature, and the maximal change in body temperature in the ensuing 2 hours, noted. The significance of drug-induced changes was calculated using the method of group comparison (13).

All drugs except reserpine were administered as salts; chlorpromazine hydrochloride, ergotamine and lysergic acid diethylamide tartrates, Hydergine methanesulphonate, serotonin creatinine sulphate, and sodium 2,4-dinitrophenol. Dosages are expressed as the free base or acid, and were selected as adequate to produce readily measurable changes in body temperature. They do not necessarily represent minimal effective doses. Drugs were dissolved in the following vehicles: chlorpromazine, serotonin, and dinitrophenol in 0.9% sodium chloride; ergotamine and LSD-25 in 0.5% tartaric acid; and reserpine in a vehicle consisting of benzyl alcohol, citric acid, polyethylene glycol 300, and water. Controls were injected with the appropriate vehicle in volumes equivalent to those administered to treated animals.

Results

The type of data obtained is illustrated in Fig. 1, which shows the results of representative experiments in which chlorpromazine (22 mg/kg) was administered to two different rats exposed to room temperatures of 30 and 39° C. Each animal was used as its own control during a separate equal period at the same ambient temperature. Chlorpromazine evoked a hyperthermia at 39° C and a hypothermia at 30° C.

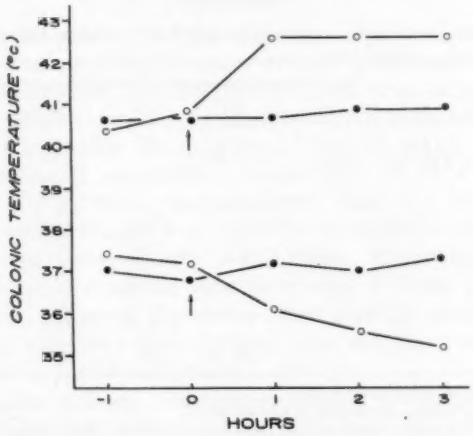

FIG. 1. Effects of chlorpromazine on body temperature of lightly restrained rats at ambient temperatures of 30° C (lower) and 39° C (upper). Drug (22 mg/kg) injected subcutaneously at zero time. Drug response (○–○) and control values (●–●) at each temperature determined in the same animal. Maximal drug-induced effects at 1 and 3 hours, upper and lower records respectively.

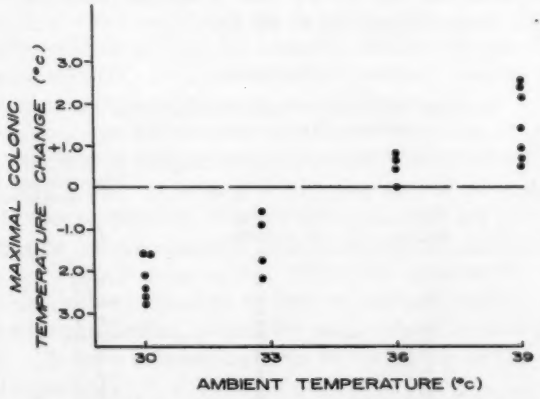

FIG. 2. Relation of ambient temperature to thermal responses of lightly restrained rats to chlorpromazine (22 mg/kg subcutaneously). Each point represents the maximal drug-induced change in body temperature in a single animal.

The maximal drug-induced changes in body temperature for all rats treated with chlorpromazine are presented in Fig. 2. The drug produced hypothermia at ambient temperatures of 30 and 33° C; no significant effect at 36° C; and significant hyperthermia at 39° C.

The average maximal changes in body temperature produced by each of the agents studied at ambient temperatures ranging from 18 to 39° C are shown in Table I. Dinitrophenol evoked a significant hypothermia at an

TABLE I

THERMAL RESPONSES OF LIGHTLY RESTRAINED RATS AT VARIOUS AMBIENT TEMPERATURES

Agent	Dose, mg/kg	Mean drug-induced changes in colonic temperature, ° C						
		18°	20°	23°	25°	30°	33°	39°
Dinitrophenol	20	−1.6 <.01* (6)†	−0.8 >.05 (7)	+2.3 <.01 (4)		+1.6 <.01 (7)		
Chlorpromazine	22					−2.2 <.01 (6)	1.4 <.05 (4)	+1.5 <.01 (7)
Hydergine	0.9	−0.9 <.02 (5)			−1.1 <.01 (5)	−0.2 >0.3 (10)	+0.6 <.02 (10)	
Ergotamine	4.0	−1.2 >.05 (5)			−1.9 <.01 (8)	+0.8 >.05 (12)	+2.0 <.01 (4)	
Serotonin	3.0				−2.2 <.01 (8)	+0.4 >.05(13)	+1.5 <.01 (7)	
LSD-25	0.5				−1.0 <.01 (8)	+0.2 >.05 (9)	+0.8 <.05 (9)	
Reserpine	1.0			−1.4 <.05 (5)	0.0 (10)	+0.6 <.01 (10)	+0.5 >0.2 (10)	+0.3 >0.2 (9)

*P value of significance of drug-induced change.
†Figures in parentheses indicate the number of animals used.

ambient temperature of 18° C, no significant change at 20° C, and a significant hyperthermia at 23 and 30° C. Hydergine, ergotamine, serotonin, and LSD-25 all produced a significant hypothermia at 25° C, no consistent effect at 30° C, and a significant hyperthermia at 33° C. Reserpine caused a significant hypothermia at 23° C, but produced somewhat inconsistent effects at higher temperatures (up to 39° C).

TABLE II

THERMAL RESPONSES OF CURARIZED RATS AT AN AMBIENT TEMPERATURE OF 16° C

Agent	Dose, mg/kg	No. of animals	Mean change in colonic temperature*	
			° C	±S.E.
Control	—	6	−6.5	±0.5
Dinitrophenol	20	6	−6.7	±0.5
Chlorpromazine	22	7	−8.5	±0.4
Hydergine	0.9	8	−6.0	±0.3
Ergotamine	4.0	6	−6.7	±0.2
Serotonin	3.0	6	−6.4	±0.3
LSD-25	0.5	6	−6.8	±0.3
Reserpine	1.0	6	−6.1	±0.3

*Measured over 2-hour period following drug injection.

The effects of various agents on the body temperature of curarized rats under artificial respiration in a room maintained at $16 \pm 1°$ C are shown in Table II. The average fall in body temperature over a 2-hour period following drug injection is recorded. Chlorpromazine was the only agent that elicited a significantly greater hypothermia than that observed in the control animals. The body temperature of the chlorpromazine-treated group decreased an average of 8.5° C, whereas that of the control group fell 6.5° C ($P < 0.01$).

Discussion

The results of this study show that ambient temperature is a critical factor determining thermal responses to many drugs. Investigation of a variety of agents has demonstrated the existence for each drug of a *critical ambient temperature*, above which hyperthermia occurred and below which hypothermia was evoked. One possible exception was reserpine, which produced a consistent hypothermia at an ambient temperature of 23° C, but somewhat inconsistent effects at higher temperatures. Bein (8) reported reserpine-induced hyperthermia in rabbits at higher room temperatures, but his limited data did not reveal the consistency of this effect. It is possible that the slow onset of the effects of reserpine may have been a factor in the inconstant results obtained. However, the effects at an ambient temperature of 23° C were consistent, and the relatively large dose employed should have been effective within the 3-hour test period.

Because changes in body temperature depend on an imbalance of heat production and heat loss, ambient temperature may influence the net thermal response to a drug via several mechanisms. Both heat loss and heat production depend to a large degree on environmental temperature. For example, drug-induced enhancement of heat loss is minimized by high ambient temperatures, because of the reduced gradient between skin and external environment. This effect would be a continuous function of ambient temperature. On the other hand, a drug which suppresses the thermogenetic response to cold would affect body temperature only in the range of ambient temperatures in which this response is elicited. It is obvious that ambient temperature also will affect responses to any agent inducing poikilothermia.

The effect of ambient temperature on thermal responses to drugs noted in the present investigation suggests that these agents interfere with central body temperature regulation. It is only within the central nervous system that the various factors involved in temperature regulation are integrated, and it is in this area that defenses against both hyperthermia and hypothermia can be impaired by a single drug action. Even the response to dinitrophenol, an agent which has been assumed to have a predominantly peripheral effect of increasing heat production, is modified qualitatively by changes in ambient temperature. (A more detailed analysis of the action of dinitrophenol will be published separately.)

It is interesting that the critical ambient temperatures for four of the drugs included in the present investigation lie in the range of thermal neutrality

for the rat. In this range of environmental temperature the homothermic animal maintains a constant body temperature with minimal physiological control. A critical ambient temperature of 30° C for Hydergine, ergotamine, serotonin, and LSD-25 suggests that these drugs induce poikilothermia, probably by an action on areas in the hypothalamus. The fact that these agents all appear to produce hypothermia by an effect on skeletal muscle tone or activity, as evidenced by their failure to act in curarized rats, is consistent with this interpretation. The thermogenetic effect of increased skeletal muscle activity is the most important defense against cold in homothermic animals, and is under central nervous system control (14).

In contrast to the other drugs studied, chlorpromazine induced hypothermia in curarized animals. This action and the production of hypothermia at ambient temperatures above thermoneutrality suggest that factors other than central suppression of skeletal muscle thermogenesis are involved in its hypothermic action. Chlorpromazine does not depress basal metabolism (15,16,17) but has been shown to produce cutaneous vasodilatation (18,19) and it appears probable that enhanced heat loss is a major component of its hypothermic effect. The high critical ambient temperature for chlorpromazine (about 36° C) may be the resultant of enhanced heat loss superimposed on impairment of central temperature regulation. Similarly, it appears probable that the low critical temperature for dinitrophenol is determined by the combined effects of impaired temperature regulation and increased peripheral heat production.

Deming *et al.* (20) claimed that the hypothermic action of reserpine is mediated through serotonin and it has been implied by Brodie *et al.* (21) that the central effects of LSD-25 are a result of serotonin antagonism. The present studies offer no conclusive evidence concerning the relationship of reserpine and serotonin in their effects on body temperature, although it is clear that responses to the two agents are not identical. However, the finding that LSD-25 and injected serotonin produce qualitatively identical effects on body temperature at various ambient temperatures indicates that serotonin antagonism cannot be invoked as the mechanism of the thermal responses of rats to LSD-25.

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THE DEGRADATION OF CALF THYMUS DEOXYRIBONUCLEIC ACID BY PANCREATIC DEOXYRIBONUCLEASE[1]

R. O. HURST

Abstract

The hydrolysis of deoxyribonucleic acid by pancreatic deoxyribonuclease was studied using high concentrations of enzyme. An increased production of material soluble in uranyl acetate reagent was obtained. Evidence for heterogeneity in the activity of the enzyme is presented.

Introduction

The action of pancreatic deoxyribonuclease (DNA-ase) on calf thymus deoxyribonucleic acid (DNA) results in the hydrolysis of DNA to a complex mixture of oligonucleotides (1, 2). Little and Butler (3) indicated that, on completion of enzymic hydrolysis of DNA to products soluble in 10% trichloroacetic acid, the liberation of secondary phosphoryl groups amounted to 25% of the total phosphorus. The inability of DNA-ase to effect the hydrolysis of all the phosphodiester linkages in DNA has not been adequately explained. In the course of previous studies of the action of DNA-ase on DNA (4) it was observed that 10 to 15% of the total oligonucleotide phosphorus was soluble in the uranyl acetate reagent used by Hurst, Little, and Butler (5) and that the dinucleotides liberated by the enzymic hydrolysis of DNA corresponded to this amount. It therefore seemed reasonable to use the uranyl acetate reagent as a means of determining more precisely the extent of the action of DNA-ase on DNA and the factors affecting it.

Materials and Methods

Deoxyribonucleic Acid

This was prepared from calf thymus by the method of Marko and Butler (6).

Deoxyribonuclease

Two preparations of DNA-ase were used: a lyophilized product, prepared according to the method of McCarty (7), which was generously supplied by Dr. G. C. Butler, and a once-crystallized preparation obtained from the Worthington Biochemical Corporation, Freehold, New Jersey. Unless otherwise noted the lyophilized preparation of DNA-ase is the one referred to in the experimental section.

Phosphorus Determinations

Total phosphorus determinations were made by a modification of the method of Beveridge and Johnson (8). Instead of preparing fresh solutions of the unstable molybdate – hydrazine sulphate reagent, the standard reagents,

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Contribution from the Department of Biochemistry, Queen's University, Kingston, Ontario. This work was supported by a grant from the National Research Council of Canada.

prepared as described by Beveridge and Johnson (8), were added separately to the digestion flask, using 5 ml of the 0.25 *N* sodium molybdate – 3 *N* sulphuric acid reagent and 2 ml of the 0.15% hydrazine sulphate reagent in that order. These two reagents keep indefinitely when stored at room temperature. The task of transferring samples after digestion was eliminated by using specially prepared glass-stoppered digestion flasks calibrated to 50.00 ml. These were obtained from Scientific Glass Apparatus Company, Bloomfield, New Jersey. The modifications suggested here overcome the criticisms against this method raised by Dryer, Tammes, and Routh (9).

Acid-soluble phosphorus (ASP) determinations were based on the solubility of oligonucleotide material in 10% trichloroacetic acid and were conducted according to a modification of the procedure of Little and Butler (3). A 1.00-ml sample was added to a 15-ml centrifuge tube containing 1.00 ml of 0.10 *N* hydrochloric acid solution and 2.00 ml of 20% trichloroacetic acid solution. After the sample was mixed by rotation, it was allowed to stand for 5 minutes at room temperature and then centrifuged for 6 minutes at 4000 r.p.m. The supernatant solution was decanted through Number 50 Whatman paper, 2.00 ml of the filtrate was diluted to 10.0 ml, and 2.00-ml samples were taken for total phosphorus analyses.

Uranyl-acetate-soluble phosphorus (USP) determinations were carried out according to a modification of the method of Hurst, Little, and Butler (5). A 1.00-ml sample was added to a 15-ml centrifuge tube containing 1.00 ml of 20% trichloroacetic acid and 2.00 ml of 1.56% uranyl acetate – 10% trichloroacetic acid reagent. The sample was mixed by rotation and allowed to stand at room temperature for 10 minutes with additional mixing and then centrifuged for 3 minutes at 2000 r.p.m. The supernatant solution was decanted through Number 44 Whatman paper and 2.00 ml of the filtrate was diluted to 10.0 ml and 2.00-ml samples were taken for total phosphorus analyses.

Hydrolysis of DNA by DNA-ase

Samples of DNA (25 mg) were dissolved in 5.0 ml of 0.10 *M* Tris (tris-(hydroxymethyl)aminomethane) buffer. These solutions were adjusted to appropriate concentrations of magnesium ion by addition of 0.20 *M* magnesium acetate and to appropriate concentrations of manganous ion by addition of 0.20 *M* manganous acetate. Merthiolate was added to a final concentration of 0.01% and the volume was adjusted to 7.0 ml with water. When ethylenediaminetetraacetic acid (EDTA) was included in the enzyme digest, it was added to the stock solution of metal ion and the pH adjusted to the value required. The enzyme was dissolved in freshly redistilled water to an appropriate concentration and 1.0 ml of this solution was added to the digest dropwise over a period of 1 minute. The enzyme digests were carried out in glass-stoppered test tubes stored in a water bath maintained at 37° C. Samples were withdrawn for the estimation of total phosphorus, phosphate soluble in 10% trichloroacetic acid (ASP), and phosphate soluble in the uranyl acetate reagent (USP).

Results and Discussion

The action of DNA-ase on DNA in the presence of 0.020 *M* magnesium ion was determined at various pH values and the results are given in Fig. 1. From the data presented it is apparent that, although the optimum pH for DNA-ase activity as measured by the production of oligonucleotides soluble in 10% trichloroacetic acid is in the range of pH 7 as reported by McCarty (7), a new pH optimum of 8 is observed for the liberation of low molecular weight oligonucleotides soluble in the uranyl acetate reagent.

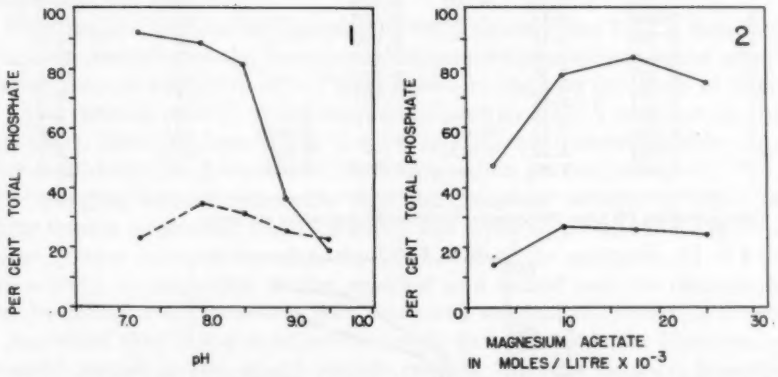

FIG. 1. The effect of pH on the hydrolysis of DNA by DNA-ase in the presence of 0.020 *M* magnesium acetate in 0.1 *M* Tris buffer at 37° C. Concentration of DNA is 3.12 mg/ml and of DNA-ase, 0.017 mg/mg of DNA. ———o———, ASP at 30 minutes; — — — ● — — —, USP at 48 hours.

FIG. 2. The effect of magnesium acetate concentration on the hydrolysis of DNA by DNA-ase in 0.1 *M* Tris buffer at pH 8 and 37° C. Concentration of DNA is 3.12 mg/ml and of DNA-ase, 0.024 mg/mg of DNA. ———o———, ASP at 15 minutes; ———●———, USP at 24 hours.

In Fig. 2 are presented the results obtained in determining the effect of the concentration of magnesium ion on the hydrolysis of DNA by DNA-ase at pH 8. Under the conditions employed in the experiments recorded in Figs. 1 and 2 it is evident that a marked increase in the production of oligonucleotide phosphorus soluble in the uranyl acetate reagent is obtained as compared to the values reported previously by Hurst (4).

The increase in the production of low molecular weight oligonucleotides at higher enzyme concentrations, as shown in Table I, indicates that DNA-ase is capable of catalyzing the hydrolysis of more phosphodiester linkages in DNA than previously considered and that the limitation of the enzyme's activity on DNA is not defined by the determination of acid-soluble phosphorus as used by Little and Butler (3). The time course of hydrolysis for several of the experiments listed in Table I is presented in Fig. 3. An initial rapid activity of the enzyme is followed by a long period of slow hydrolysis, which suggests that the length of time in these experiments is an important

TABLE I

THE EFFECT OF ENZYME CONCENTRATION, METAL IONS, AND EDTA ON THE HYDROLYSIS OF DNA BY DNA-ASE IN TRIS BUFFER, pH 8, AT 37° C

Expt. No.	DNA-ase, mg/mg of DNA	DNA, mg/ml	Mg^{++}, meq/ml	Mn^{++}, meq/ml	EDTA, meq/ml	Sample time, hours	Uranyl-acetate-soluble phosphorus, % total P
1	0.016	3.15	0.0025	—	—	24	13.8
2	0.016	3.15	0.025	—	—	24	23.6
3	0.140	3.30	0.025	—	—	18	42.2
4	0.048	3.09	0.020	0.005	—	24	38.1
5	0.048	3.15	0.005	0.020	—	21	32.5
6	0.040	3.15	—	0.005	—	21	24.9
7	0.040	3.15	—	0.020	—	18	24.2
8*	0.048	2.78	—	0.018	—	16	52.5
9*	0.107	2.50	—	0.020	—	34	77.4
10*	0.153	2.50	—	0.002	—	28	70.6
11	0.016	3.12	0.025	—	0.0125	68	6.3
12	0.016	3.12	0.025	—	0.0125	68	6.7
13	0.040	3.12	0.020	—	0.010	43	8.1
14*	0.005	3.62	—	0.006	0.003	2	20.2

*Once-crystallized DNA-ase (Worthington Biochemical Corporation) was used.

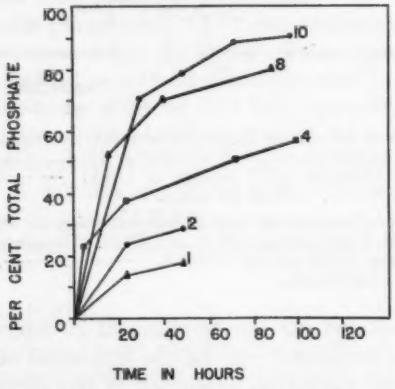

FIG. 3. Time course of hydrolysis of DNA by DNA-ase to products soluble in the uranyl acetate reagent (USP as per cent of total phosphate) in the presence of magnesium and/or manganous ions in 0.1 *M* Tris buffer at pH 8 and 37° C. Numbers refer to corresponding experiments listed in Table I.

factor. It is highly probable that much of the enzyme is denatured after the initial period of hydrolysis.

The activity of DNA-ase is dependent upon divalent cations such as magnesium or manganese ions (7) and other ions have also been reported by Miyaji and Greenstein (10) to satisfy this requirement. The fact that the enzyme is inactive in the absence of these ions suggested that the limitation in the action of the enzyme may be related to a chelation of the activating cation by the liberated secondary phosphate groups of the oligonucleotides. This would imply that the enzyme action stops because of the removal of the

necessary cations from the zone of action of the enzyme by the products formed. In order to test this hypothesis the effect of the chelating agent, ethylenediaminetetraacetic acid (EDTA), upon the enzymic reaction was studied. In preliminary studies it was found that an excess of EDTA over magnesium ion completely inhibited the ability of DNA-ase to lower the viscosity of DNA solutions and to produce material soluble in 10% trichloroacetic acid. Viberg (11) has recently reported the same observation. This inhibition is directly related to the removal of the activating ion which is bound very closely by EDTA, as indicated by the studies of Chabarek, Bersworth, and Martell (12).

When magnesium ions were present in 100% excess of the EDTA, however, a marked inhibition of the liberation of oligonucleotides soluble in the uranyl acetate reagent was obtained. This is shown by the data presented in Table I. The removal of 50% of the magnesium ions by EDTA does not explain this effect, since the data in Fig. 2 show 26.7% uranyl-acetate-soluble phosphorus produced in 24 hours at 0.01 *M* magnesium ion concentration.

It therefore seemed reasonable that the diesterase activity of DNA-ase might require some other metal ion which was probably present in the enzyme digest in trace amounts bound to the DNA. Since the optimum pH of 8 for this activity corresponded to that reported as a second peak for manganous ions by Miyaji and Greenstein (10), this metal ion was also investigated and it was found that it was equally effective as an activator in the liberation of material soluble in the uranyl acetate reagent. In this instance, however, the diesterase activity was not inhibited when the manganous ions were in 100% excess of the EDTA.

In order to test the possibility that the phosphodiesterase activity in crude DNA-ase is due to the presence of another enzyme function, once-crystallized DNA-ase was studied and was found to be even more effective than the lyophilized preparation in the production of oligonucleotides soluble in the uranyl acetate reagent, as shown by the data presented in Table I and Fig. 3. This is due to the greater unit activity of the crystalline preparation as compared to the crude, lyophilized enzyme. Colvin, Smith, and Cook (13) have drawn attention to the fact that many crystalline protein preparations are heterogeneous, not only in biological activity, but in physical and chemical properties as well. It is apparent that purification of DNA-ase by crystallization has not eliminated the diesterase function responsible for the increased degradation of DNA.

The suggestion that the enzyme preparation is heterogeneous, having the dual function of a depolymerase and a phosphodiesterase, is a possible explanation for the results obtained. Previously it has been considered that the depolymerization of DNA in solution with the consequent decrease in viscosity has been due to the hydrolysis of covalent bonds with the spontaneous rupture of the secondary valence bonds responsible for the macromolecular properties of DNA (14). The formation of acid-soluble oligonucleotides would accordingly require a much more extensive degradation of the DNA.

This is reflected in the large difference in the amounts of DNA-ase required for investigating the enzymic hydrolysis of DNA by the viscosimetric method and by the acid-soluble phosphorus method as reported by Little and Butler (3). The use of larger amounts of DNA-ase brings about a still greater degradation of the DNA.

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VARIATIONS IN THE SODIUM AND POTASSIUM CONTENT OF THE MUSCLE TISSUE OF PACIFIC SALMON WITH PARTICULAR REFERENCE TO MIGRATION[1]

ROBERT A. MACLEOD, R. E. E. JONAS, AND J. R. MCBRIDE

Abstract

Further confirmation was obtained of the observation made previously that the Na^+ and K^+ levels of the muscle tissue of spring salmon (*Oncorhynchus tschawytscha*) on the Pacific coast appear to vary with the size of the fish and, in the case of small fish, with the season of the year. Small fish (3 to 5 lb) caught in regions of relatively constant salinity had higher Na^+ and lower K^+ concentrations in the spring and summer than in fall and winter. Under the same conditions the levels of Na^+ and K^+ in the flesh of larger fish (9 to 10 lb) did not vary.

When spring salmon were sampled along the route of their spawning migration up the Fraser River it was found that at the mouth of the river the Na^+ level was less than in fish at sea. Further up the river, Na^+ had dropped to less than one-half of the value at sea. At the spawning grounds in three groups out of four examined, Na^+ rose again to levels the same as or above those prevailing at sea. In general, a drop in Na^+ was accompanied by a rise in K^+ and vice versa, though at the spawning ground the drop in K^+ considerably exceeded the rise in Na^+.

In the case of sockeye salmon (*Oncorhynchus nerka*) heading up river, a lowered Na^+ and elevated K^+ occurred in the muscle tissue of the fish while they were still in salt water. After the fish had entered fresh water the ion levels were restored and maintained at levels similar to those normally prevailing at sea. At or near the spawning ground a marked rise in Na^+ as well as a drop in K^+ occurred. Evidence was obtained that this change occurred first in the male of the species.

The flesh of juvenile coho salmon (*Oncorhynchus kisutch*) migrating to the sea was found to contain less Na^+ and more K^+ than mature fish of the same species caught at sea. Young sockeye in fresh water, however, contained about the same levels of Na^+ and K^+ as the mature fish at sea.

Introduction

Fish which migrate from fresh water to sea water or from sea water to fresh water or both face very special problems in osmoregulation. In sea water the osmotic pressure of the medium is greater than that of the body fluids. To maintain the water and salt balance in sea water, teleost fish drink sea water and excrete excess inorganic ions through the gills and by way of the urine. In fresh water the reverse holds. Excess water is excreted by an increased urine flow and salts are conserved. For a review see Black (4).

It has been found that adult salmon returning to fresh water exhibit a slight decrease in osmotic concentration of the blood (2,9). At the spawning grounds this decrease amounted to 17% of the concentration at sea. Eels (*Anguilla* spp.) migrate as adults from continental fresh waters to spawn in the Atlantic Ocean. Loss of body salts has been observed in these fish prior to entering the sea (5). Whether these changes occur as a result of alterations

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Contribution from Fisheries Research Board of Canada, Technological Station, Vancouver, B.C.

in the salt content of the environment or are produced by hormone effects associated with migration has not been established clearly.

In a previous investigation a survey was made of the Na^+ and K^+ content of the flesh of fish caught in commercial quantities on the coast of British Columbia (12). It was noted that in the case of salmon, considerable variations in the flesh levels of these ions occurred which appeared to depend on the size of the fish and the season when they were caught. It was speculated that the change with season might be related to the approach of the fish to the time of their spawning migration. The present study was undertaken to explore this possibility further. Samples of both spring and sockeye salmon were obtained at various points along the route of their migration up the Fraser River and their flesh levels of Na^+ and K^+ determined. The levels of Na^+ and K^+ in two species of young salmon migrating downstream to the sea have also been obtained. Marked variations in flesh levels of the ions have been observed and factors responsible for these changes considered.

Experimental

Source of Materials

Fish from the sea were obtained directly from fishing boats. Spring salmon (*Oncorhynchus tschawytscha*) caught at various points on the Fraser River were obtained through the kind co-operation both of the Department of Fisheries, Vancouver, and of Dr. D. J. Milne of the Biological Station at Nanaimo. The fish were placed individually in polyethylene bags when caught and the bags were then packed in ice for transport to the laboratory. This arrangement prevented possible leaching of the fish by the melting ice.

Sockeye salmon (*Oncorhynchus nerka*) samples were obtained through the co-operation of the International Pacific Salmon Fisheries Commission. The fish collected in 1956 were transported under the same conditions as those used for the spring salmon. In 1957, however, fish were bled and then frozen in dry ice at the site of capture. Transport of these fish to Vancouver was effected, using a portable deep-freeze unit. All of the samples of sockeye salmon were used as sources of material for other experimental work besides that recorded here. More complete details of the capture and handling of these fish are presented elsewhere (10).

Methods of Sampling and Analysis

All fish were eviscerated and then sampled according to a slight modification of the official A.O.A.C. method. Three slices were removed from each fish, one from behind the head, one from the mid-section, and one from just in front of the tail. The muscle tissue from each slice was separated from the bones and skin, pooled, and homogenized. Aliquots of the homogenized samples were solubilized with nitric acid. Analyses were performed using a flame photometer attachment for a Beckman DU spectrophotometer. Details of the procedures used have been described (12). Fat analyses were performed by the method of Damberg (7).

Results

Fish Caught at Sea

In the course of a previous investigation into the Na^+ and K^+ levels in the flesh of Pacific salmon (12), it was observed that spring salmon in the small size range (2 to 7 lb) had higher Na^+ and lower K^+ levels in the spring of the year than later in the summer. In the summer the levels approached those of fish in a larger size range (7 to 20 lb).

It was decided to reinvestigate this phenomenon for the following reasons. The sea is not a perfectly uniform environment. Areas of sea water near the shore can be diluted by fresh water from rivers. The types of food ingested by fish vary with availability from season to season and from one year to another. Since fish tend to run in schools, fish caught at any one time are not necessarily random samples but may all be representative of a group of fish which have been subjected to similar environmental conditions.

It was felt that if the types of variation observed previously were found to occur in another year, the results could be more readily attributed to physiological changes in the fish rather than to responses of the fish to transitory environmental conditions. Accordingly, fish in two size ranges were caught at different times during 1956 at points along the west coast of Vancouver Island, where the salinity of the sea water is not influenced appreciably by run-off from the Fraser River (13).

TABLE I

EFFECT OF SIZE AND SEASON ON THE Na^+ AND K^+ CONTENT OF THE MUSCLE TISSUE AND TISSUE WATER OF SPRING SALMON CAUGHT AT SEA*

Size range†	Time of catch	No. of fish	Average wt., lb	Na^+ and K^+ in fat-free tissue, meq per kg§		Na^+ and K^+ in tissue water, meq per kg	
				Na^+	K^+	Na^+	K^+
Small	July 20	3	4.94 ± .42‡	28.4 ± 3.0	112.7 ± 1.6	36.4 ± 3.9	144.2 ± 2.0
	Aug. 1	3	3.50 ± .28	23.9 ± 0.6	114.0 ± 1.1	30.8 ± 0.7	147.3 ± 1.4
	Dec. 1	3	4.94 ± .70	19.1 ± .32	127.2 ± 0.8	24.4 ± 0.4	162.2 ± 1.0
Medium	July 20	3	10.40 ± .47	19.6 ± 1.5	123.9 ± 4.0	26.0 ± 2.0	164.7 ± 5.3
	Aug. 1	3	9.31 ± .67	19.1 ± 1.0	120.7 ± 1.4	24.9 ± 1.3	157.2 ± 1.8
	Dec. 1	3	10.00 ± .67	19.6 ± 2.5	125.4 ± 5.7	25.3 ± 3.2	162.1 ± 7.4

*West coast of Vancouver Island.
†Small, 2–7 lb; medium, 7–20 lb; large, 20 lb and up.
‡Deviations shown are average deviations of the mean.
§meq per kg = milliequivalents per kilogram.

The results in Table I show that small fish caught in July had significantly higher Na^+ and lower K^+ levels than those caught in December ($P < .02$ in each case). An intermediate value was recorded in August, suggesting a downward trend for Na^+ and an upward one for K^+ between July and December. In December, the concentrations found were the same as those which had prevailed in fish in the medium size range over the whole period. In the previous study (12) the drop in Na^+ and rise in K^+ had been found to occur

between June and July. In the present case the change in levels did not begin until after the July sample was taken.

Since the reasons for the variation in the Na^+ and K^+ content of the fish with size were obscure and since the factors mentioned above could introduce so many sampling problems, other fish in two size ranges were analyzed for their Na^+ and K^+ concentrations. In this case the fish were caught during May and June since the previous study (12) had indicated that variations in the ion concentration with size could most likely be expected at this season. Nine fish in the small size range and 12 in the large were sampled and analyzed over the 2-month period. The results (Table II) show that the differences noted previously between large and small fish occurred again in this experiment. Larger fish almost invariably contain more fat in the muscle tissue than small ones as the results in Table II show. That this is not the factor

TABLE II

COMPARISON OF Na^+ AND K^+ LEVELS IN MUSCLE TISSUE AND TISSUE WATER OF SPRING SALMON IN TWO SIZE RANGES CAUGHT AT SEA*

	Size range		Significance of differences
	Small	Large	
Tissue levels‡			
Na^+, meq/kg	23.04 ± .76†	17.21 ± .68	$P<.001$
K^+, meq/kg	115.0 ± 2.9	128.1 ± 2.9	$P<.001$
Tissue water levels			
Na^+, meq/kg	28.42 ± 3.17	22.38 ± .82	$P=.05$
K^+, meq/kg	141.8 ± 9.5	166.5 ± 4.6	$P=.02$
Weight of fish, lb	4.92 ± .52	20.37 ± 1.09	
Fat content, %	2.84 ± .24	7.56 ± 0.90	
No. of fish analyzed	9	12	

*All fish caught in May and June.
†All deviations reported are standard error of the mean.
‡Based on fat-free muscle tissue.

responsible for the variation in ion levels is evident from the fact that all calculations of muscle levels of ions were made on the basis of fat-free tissue.

The findings presented here and observed previously (12) indicate that at certain times of the year the muscle tissue of small spring salmon can be expected to contain more Na^+ and less K^+ than large ones.

Fish Migrating Upstream

Salmon often travel hundreds of miles up rivers to their spawning grounds. On these journeys no food is consumed. It was of interest to know how well flesh levels of Na^+ and K^+ were maintained during upstream migration. Accordingly, arrangements were made to obtain spring salmon caught at various points on the Fraser River and at the spawning grounds.

Fresh water from the Fraser River reduces the salinity of the sea water for a considerable distance from the river mouth (13). Two groups of fish

were caught at the mouth of the Fraser and their flesh concentrations of Na^+ and K^+ compared with values obtained on fish of the same size caught at approximately the same time at a point where normal ocean salinities prevail. The results show (Table III) that the fish at the river mouth had lower Na^+ and higher K^+ concentrations than occurred in fish at sea.

At the next point taken in the course of upstream migration, a point 90 miles from the sea, the Na^+ concentration in the flesh had dropped to nearly half that at sea, while the K^+ level, except in the case of the one fish sampled, was much the same as the concentration at sea.

Fish from three different spawning grounds were sampled. Two of these, spawning grounds I and II, were 190 and 280 miles respectively from the mouth of the Fraser River, while the third was only a comparatively short distance, 60 miles, upstream. At spawning grounds I and II the Na^+ concentration had risen to levels which were even higher than those in fish at sea, while K^+ had dropped very considerably. As can be seen from the table, the changes recorded appear to be relatively independent of the size of the fish.

The fish on spawning ground III represented a later run of spring salmon. The results obtained here were compared with values from fish caught at sea at approximately the same time. At this spawning ground a random sampling of eight fish gave two groups of four which differed markedly from one another with respect to flesh Na^+ levels. In group I the muscle Na^+ concentrations were the same or somewhat higher than those of fish at sea. In group II, the low Na^+ concentrations characteristic of fish which have just recently entered fresh water were recorded. The reasons for the differences in the two groups of fish at this third spawning ground are obscure. All eight fish involved had spawned but were alive when caught. There was about an equal distribution of males and females in each group. There was no obvious correlation of the results with size.

The analysis for protein shows that the muscle tissue of the spawned fish contained only half as much protein as that of fish at sea. This indicates that since the fish do not eat, body protein plays a major role in providing energy for migration as well as substrate for gonad development. Such an observation has been made previously (9). The loss of muscle protein and hence of muscle tissue cells could account for the drop in muscle K^+ concentration. If cells are being replaced by extracellular water, one might also expect a rise in tissue Na^+. That this is not the only explanation for the increase in tissue Na^+ at the spawning ground, however, is indicated by the fact that both groups of fish at spawning ground III have similarly low muscle protein levels, but considerably different tissue Na^+ concentrations.

Mature sockeye salmon differ from spring salmon in being of a very uniform size (5 to 7 lb). A number of different runs of this species of salmon each consisting of a relatively pure race of fish having a particular spawning ground as their goal can be distinguished each year on the Fraser River (11). One of these runs was sampled in 1956. The results are recorded in Table IV. In this study three fish were obtained at each of the points shown. Since the

TABLE III

COMPARISON OF Na^+ AND K^+ LEVELS IN MUSCLE TISSUE AND TISSUE WATER OF SPRING SALMON CAUGHT AT VARIOUS POINTS ON THE COURSE OF THEIR UPSTREAM MIGRATION

Location	No. of fish	Size	Na^+ and K^+ in fat-free tissue, meq/kg		Na^+ and K^+ in tissue water, meq/kg		H_2O, %	Protein, %
			Na^+	K^+	Na^+	K^+		
At sea*	3	Medium	19.1 ± 0.7**	120.7 ± .8	24.9 ± .91	157.2 ± 1.1	71.3	—
River mouth†	3	Medium	15.4 ± 0.3	134.0 ± 4.1	21.1 ± .45	183.0 ± 5.6	68.1	—
	2	Medium	14.0 ± 3.3	128.7 ± 4.6	18.4 ± 4.3	170.0 ± 6.2	70.7	—
Upriver 90 miles‡	1	Medium	6.76	103.6	8.4	129.5	77.2	—
Upriver 90 miles	2	Small	9.62 ± .51	122.7 ± 1.4	12.6 ± .67	160.5 ± 1.8	71.9	—
Spawning ground I§	3	Small	22.7 ± 0.3	109.3 ± 0.8	28.5 ± 0.4	137.5 ± 1.0	76.1	—
Spawning ground II‖	4	Small	23.6 ± 3.9	86.3 ± 7.0	28.0 ± 4.6	102.3 ± 8.3	83.2	—
Spawning ground II	4	Medium	26.2 ± 4.4	87.0 ± 8.0	31.6 ± 5.3	105.0 ± 9.7	81.8	—
At sea*	1	Small	19.0	127.9	24.3	162.9	73.5	19.5
	1	Medium	19.7	126.3	25.1	160.8	74.6	19.2
Spawning ground III¶								
Group I	4	—	21.0 ± 2.0	80.8 ± 2.3	23.7 ± 2.3	91.1 ± 3.3	88.3 ± .7	10.3 ± .9
Group II	4	—	11.6 ± 1.4	89.3 ± 6.3	13.1 ± 1.6	101.3 ± 7.3	87.7 ± 1.3	11.2 ± 1.0

*West coast of Vancouver Island; †Fraser River; ‡Hope, B.C.; §Seton Creek, up the Fraser River 190 miles.
‖Adams River, up the Fraser River 280 miles; ¶Harrison River, up the Fraser River 60 miles.
**Deviations reported are average deviations of the mean.

TABLE IV

CHANGES IN THE LEVEL OF Na^+ AND K^+ IN THE MUSCLE TISSUE AND TISSUE WATER AND IN THE WATER, FAT, AND PROTEIN CONTENT OF THE MUSCLE TISSUE OF SOCKEYE SALMON DURING UPSTREAM MIGRATION (STUART RUN, FRASER RIVER, 1956)

Location	Miles upriver*	Na^+ and K^+ in fat-free tissue, meq/kg		Na^+ and K^+ in tissue water, meq/kg		H_2O, %	Fat, %	Protein, %
		Na^+	K^+	Na^+	K^+			
San Juan Is.	At sea	19.49 ± 1.49§	120.0 ± 0.7	26.30 ± 1.94	162.0 ± 1.4	69	6.2	23.1
Lummi Island	135†	13.48 ± 0.59	128.5 ± 0.7	18.16 ± 0.76	173.2 ± 1.2	69	6.6	22.7
Albion	215	17.82 ± 0.76	113.7 ± 1.4	23.79 ± 1.05	151.8 ± 1.7	71	5.2	22.4
Hell's Gate	305	17.54 ± 1.31	118.3 ± 2.5	23.13 ± 1.65	156.0 ± 3.8	73	3.9	22.2
Lillooet	385	17.05 ± 2.08	113.7 ± 1.5	22.52 ± 2.52	150.3 ± 3.3	73	3.7	21.5
Soda Creek	515	18.51 ± 0.80	110.0 ± 3.2	24.18 ± 1.00	143.7 ± 4.3	75	2.5	21.4
Fort St. James	775	26.36 ± 4.10	96.1 ± 2.7	32.81 ± 5.04	119.8 ± 4.2	79	1.6	18.4
Forfar Creek‡	850	31.54 ± 2.06	94.2 ± 0.9	39.55 ± 2.85	118.0 ± 0.6	79	1.3	19.2

*Up the Fraser River from San Juan Island.
†This point is still in sea water.
‡Spawning ground.
§Deviations recorded are average deviations of the mean.

fish were to be used in other investigations as well, one side only of each was taken for analysis. Sampling was conducted in the usual way, however, by pooling the skin-free, bone-free muscle tissue from three slices from each fish. It can be seen from the results that in the case of sockeye salmon the drop in Na^+ occurred even before the fish entered fresh water. For a good part of the distance up the river the Na^+ levels were very nearly the same as those of the fish at sea. Over the last one-third of the journey the Na^+ levels rose until, at the spawning grounds, they were 75% higher than the values at sea. During this latter part of the journey a progressive decline in K^+ occurred.

In view of the difference in Na^+ concentration with sex at the spawning ground which will be described in connection with the next table (Table V), it is of interest to consider the sex of the fish analyzed in this experiment. The three fish sampled at Fort St. James (Table IV) consisted of two females and one male. The male fish had the high tissue Na^+ concentration of the fish at Forfar Creek while the two female fish had levels similar to those prevailing in the fish at Soda Creek. The three fish at Forfar Creek spawning ground also consisted of two female and one male fish. In this case, however, the two female fish had the same high tissue Na^+ concentrations as the male.

It should also be noted that the percentage rise in tissue Na^+ level of the sockeye salmon at the spawning ground was considerably greater than was observed in the case of the spring salmon. On the other hand, the protein loss from the muscle tissue of the sockeye salmon was very much less than occurred in the spring salmon. These species differences lend further support to the conclusion reached previously that it is unlikely that the rise in tissue Na^+ at the spawning ground is due primarily to replacement of muscle tissue cells by extracellular water.

Samples of the same run of sockeye were obtained the following year (1957). In this case a much larger number of fish were sampled. Four groups of males and four groups of females, each group containing 8 to 10 fish, were obtained from each of two points, Lillooet and the spawning ground, Forfar Creek. Each fish was sampled by removing a 1-in. cube of flesh from left side of the back immediately ahead of the dorsal fin. These fish differed also from those studied previously in that they had been bled prior to sampling. Comparison of the Na^+ values at the two points (Table V) show that the greatest rise in Na^+ occurred in the males, though a significant rise in this ion (significance of difference, $P < .05$) also occurred in the females. In both cases the K^+ levels dropped considerably.

Young Salmon in Fresh Water

Because of the marked variations in the concentration of Na^+ and K^+ in the flesh of adult salmon it was of interest to know what levels existed in young salmon found in fresh water. Juvenile sockeye and coho salmon (*Oncorhynchus kisutch*) which were either in the process of migrating downstream or due shortly to begin the trip to the sea were obtained for analysis.

TABLE V

COMPARISON OF THE Na^+ AND K^+ LEVELS IN THE MUSCLE TISSUE AND TOTAL TISSUE WATER OF MALE AND FEMALE SOCKEYE SALMON AT TWO POINTS ON THE FRASER RIVER STUART RUN (1957)

Location of catch	Miles upstream	Sex	Na^+ and K^+ in fat-free tissue,* meq/kg		Na^+ and K^+ in tissue water, meq/kg	
			Na^+	K^+	Na^+	K^+
Lillooet	385	Male	12.98 ± .21†	118.6 ± 0.8	17.26 ± .21	157.8 ± 1.4
		Female	13.42 ± .26	117.2 ± 1.2	17.73 ± .31	154.8 ± 1.3
Forfar Creek (spawning ground)	850	Male	30.98 ± 2.69	102.0 ± 2.7	38.63 ± 3.35	127.2 ± 2.1
		Female	16.16 ± 0.39	111.6 ± 0.9	20.26 ± 0.46	140.0 ± 1.8

*Based on fat-free skeletal muscle tissue. In this experiment only, the fish were bled immediately after capture.
†Deviations recorded are standard errors of the mean.

TABLE VI

Na^+ AND K^+ IN THE MUSCLE TISSUE AND TOTAL TISSUE WATER OF JUVENILE SALMON OBTAINED FROM FRESH WATER

Species	Source*	No. of samples†	Na^+ and K^+ in fat-free tissue, meq/kg		Na^+ and K^+ in tissue water, meq/kg	
			Na^+	K^+	Na^+	K^+
Coho	Capilano River	4	10.34 ± .93‡	123.0 ± 1.5	12.59 ± 1.13	149.7 ± 1.9
Coho	Cultus Lake	3	16.19 ± 0.18	145.6 ± 1.3	20.43 ± .22	183.7 ± 1.6
Sockeye	Cultus Lake	3	24.56 ± .43	106.9 ± 2.1	–	–

*Analysis of H_2O samples: Capilano River, Na^+ .039, K^+ .026 meq/kg. Cultus Lake Na^+ .248, K^+ .026 meq/kg; by comparison, sea water contains approximately Na^+ 457, K^+ 9.75 meq/kg.
†Because of the small size of each fish, each sample consisted of flesh obtained by pooling skin- and bone-free portions of 10 to 15 fish.
‡Deviations recorded are average deviations of the mean.

Table VI shows that the flesh of coho salmon from one source had extremely low Na^+ levels, while those from another were considerably higher, though still well below the average for mature fish of this species at sea (23–24 meq/kg) (12). Corresponding K^+ levels were above the average for these fish at sea. In the case of the sockeye, the Na^+ value obtained was even slightly higher while K^+ was about the same as the averages for mature fish of this species at sea. That these levels bear no obvious relation to the content of Na^+ and K^+ in the water in which the fish were caught is apparent from the results of analyses which are recorded beneath Table VI.

Discussion

The findings presented here and observed previously (12) indicate that at certain times of the year the muscle tissue of small spring salmon contains more Na^+ and less K^+ than that of larger fish. The reason for this is obscure. Barlow and Manery (1) found that the muscle tissue of young chicks contained more Na^+ and less K^+ than that of adults and concluded that this change with age was due to the displacement of extracellular fluid by cells. Since

small salmon are in most cases also young salmon, a similar situation could conceivably exist in the case of small and large fish. Since the elevated Na^+ and lowered K^+ in small salmon occurred only at certain seasons of the year, however, it is difficult to attribute to maturation the establishment at other seasons of levels prevailing in larger fish.

During upstream migration the changes in Na^+ and K^+ were somewhat different in the two species of salmon examined. In the case of sockeye, a lowered Na^+ and elevated K^+ occurred in fish still in salt water (Lummi Island), suggesting that in this species the initial change observed may not be a consequence of the fish entering fresh water but a reflection of a change in hormone levels associated with migration. After this preliminary change, even though the fish entered fresh water, the ion levels were restored almost to the levels which normally prevailed at sea. These concentrations were then maintained until the spawning grounds were either reached or closely approached.

Whether, in the case of spring salmon, ion changes occurred in the tissues before or after the fish had entered fresh water cannot be stated. There was not the same opportunity to follow a group of spring salmon from salt water to fresh water as existed with the run of sockeye salmon. Once the fish were in fresh water, however, much more drastic reductions in Na^+ level occurred in spring salmon than sockeye and these obtained for much longer distances up the river.

At the spawning grounds an unusual and unexpected situation prevailed. At all of the spawning grounds tested and for both species of salmon, fish were found which had elevated Na^+ and lowered K^+ concentrations in the muscle tissue. Although this would appear to be the general trend of ion changes at the spawning ground, two exceptions to this pattern were noted. One group of spring salmon at one of the spawning grounds showed no elevation in tissue Na^+ though this group appeared to be in exactly the same physiological state as another group which did display the high Na^+ concentration. For sockeye salmon, in the 1957 experiment, the marked rise in Na^+ level occurred only in the male.

No explanation can be offered for the variation in tissue Na^+ concentration in spring salmon at the third spawning ground. For the sockeye salmon there is an apparent discrepancy between the results of the 1956 and 1957 experiments in regard to the rise in tissue Na^+ in the female fish at the spawning ground. In the 1956 experiment both male and female fish showed high tissue Na^+ levels at the spawning ground while in 1957 the steep rise was recorded only in the male. A possible explanation for this discrepancy lies in the fact that this particular run of fish does not spawn immediately after the fish have reached the spawning grounds but rather about a week after the fish have arrived. During this week, the final stages in gonad development apparently occur. If the rise in tissue Na^+ takes place in male fish before it occurs in female fish and if the runs in the two successive years were not sampled at the spawning ground at a time when the fish were in exactly the

same stage of physiological development, the apparent discrepancy in results could occur. In support of this possibility is the indication in the 1956 sockeye experiment that the rise in tissue Na^+ occurred in the male fish even before the spawning ground was reached, though insufficient numbers of male and female fish were included in the 1956 samples to permit any definite statement to be made. The fact that a small but significant rise in muscle Na^+ had occurred in the female fish at sampling time in the 1957 experiment lends further support to the explanation offered. In addition, in the 1957 study, the last sample was taken immediately after the fish had arrived at the spawning ground, so that approximately a week would have remained for further changes in the tissue Na^+ concentration of the female to have taken place.

The drop in tissue K^+ concentrations in spring salmon at the spawning grounds is much greater than for sockeye salmon and this species difference is roughly in proportion to the much lower muscle protein concentration in the spring salmon. A loss of muscle tissue cells through starvation could thus be largely responsible for the loss in tissue K^+. That it is not entirely responsible is suggested by the fact that the drop in tissue K^+ in sockeye salmon in the 1957 experiment, Table V, was greater in the male fish, though protein losses from the muscle have been found to be appreciably greater in the female fish at this point owing to the fact that in the latter the gonads developed represent a much larger proportion of the total body weight (10).

That the loss of muscle tissue cells and its replacement by extracellular water is unlikely to be responsible for the marked rise in tissue Na^+ observed at the spawning grounds has already been considered at various points in the text.

In all cases observed at the spawning ground the drop in K^+ exceeded the rise in Na^+ and the sum of the Na^+ and K^+ concentrations in the tissue water was less than was found at sea. Since there is an increased percentage of water in the flesh of fish at the spawning ground, it might at first appear that the lowered electrolyte concentration was due to dilution. Another investigation has shown, however, that for the sockeye salmon there is only an approximately 10% increase in the volume of muscle tissue water in the male and an actual decrease in the female during migration (10). The increased percentage of water recorded in Table IV then is due largely to a reduction in the amount of protein and fat in the tissue rather than to a gain in tissue water.

The sharp rise in Na^+ and at least part of the drop in K^+ which has been observed to occur in the tissues of the two species of salmon at the end of the migration could conceivably be due to heightened hormone activity associated with the final stages of sexual development before spawning. A similar rise in muscle Na^+ and drop in K^+ occurred in brown trout as a result of an injection of 11-deoxycorticosteroids (6) and it is the same type of response which has been obtained from an injection of these hormones in mammals. So far, however, this particular group of hormones has not been shown to occur in fish. In elasmo-branchs, changes in interrenal activity have been

detected during the sexual cycle (8) though in this group of fish cortical steroids appear to affect carbohydrate rather than mineral metabolism (3).

The Na^+ and K^+ levels found in the flesh of coho and sockeye fingerlings migrating to sea indicate that the sockeye differ from the coho in having flesh levels of the ions very much like those of mature fish of the same species at sea. It may be of significance in this connection that sockeye can, if necessary, spend all their life in fresh water while coho must migrate to the sea to survive.

The results recorded here show that both at sea and in fresh water wide variations in flesh levels of Na^+ and K^+ occur in two species of Pacific salmon. They also indicate that such factors as size of the fish, season of the year, salinity of the water, sex of the fish, and probably also hormone interrelationships, may determine the concentrations of these ions in the flesh. To understand better the mechanism of the changes which occur, considerably more information about such factors as ion changes in the blood of the fish, distribution of ions between intracellular and extracellular water in the flesh, hormone changes, and the influence of these on electrolyte metabolism in fish are required. Since most of the phenomena investigated here cannot be reproduced in the laboratory it is necessary to obtain samples far from normal laboratory facilities. The only samples on which reliable Na^+ and K^+ determinations could be made with present facilities were samples of whole flesh. When mobile laboratory equipment permitting studies at the site of capture of the fish becomes available, a more searching analysis of some of the factors which have been brought to light in this investigation will be made.

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STUDIES ON THE PRESERVATION OF BLOOD

VI. THE INFLUENCE OF ADENOSINE AND INOSINE ON THE METABOLISM OF THE ERYTHROCYTE[1]

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With the technical assistance of SHEILA M. GOSSELIN

Abstract

Inosine is as effective as adenosine in maintaining the organic phosphate esters in the erythrocytes during storage in the citrate–dextrose preservative medium. Adenosine undergoes deamination in the presence of erythrocytes with liberation of ammonia. The ammonia tends to counteract the increase in the hydrogen ion concentration caused by the glycolytic production of lactic acid. By maintaining the hydrogen ion concentration within the range favorable to the activity of hexokinase, adenosine tends to maintain the utilization of glucose in the preserved red cells. Inosine, on the contrary, does not resist the increase in hydrogen ion concentration of the cells during storage, hence the utilization of glucose rapidly becomes impaired and supplanted by the utilization of ribose derived from the nucleoside. The utilization of ribose remains practically unaffected by the increase in hydrogen ion concentration to pH 6.1. Ammonium ions stimulate the utilization of glucose by erythrocytes but in degree not sufficient to account for the full effect of adenosine.

Introduction

Gabrio and Finch (1) in 1954 observed that the addition of adenosine to specimens of preserved blood induced a re-esterification of the inorganic phosphate that accumulates from hydrolysis of the organic phosphate esters in the red cells during storage. They showed further that the restoration of the organic phosphate compounds is accompanied by an improvement in the viability of the cells (2). Studies in our laboratory (3) showed that adenosine undergoes deamination in the red cells with the formation of inosine which, in turn, undergoes phosphorolysis to yield hypoxanthine and ribose phosphate. The hypoxanthine is not further altered in the cells but most of it diffuses into the plasma. The ribose phosphate is metabolized chiefly to lactic acid.

Inosine is metabolized as readily as adenosine by the red cells (9). It offers a definite advantage over adenosine for consideration as an adjuvant for red cell preservation in that it is nontoxic (9). The study reported here concerns certain differences observed between the metabolic behavior of the two nucleosides in the red cells during storage of blood at 4° C.

Methods

Reagents

Adenosine and inosine were obtained from Nutritional Biochemicals Corporation, Cleveland.

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Procedures

Blood was collected into the acidified citrate–dextrose (ACD) and citrate–dextrose (CD) preservative media, as described in a previous communication (3). Hemolyzates were prepared by suspending washed red cells in 2 to 3 volumes of a 0.02 *M* solution of nicotinamide. The specimens were analyzed for glucose (4), lactate (5), pyruvate (6), phosphate (7), ribose (8), nucleoside (9), and ammonia, which was determined by nesslerization of the protein-free filtrates. The fractional analysis for inorganic acid-soluble, labile (ATP), 'hydrolyzable', and stable (DPG) phosphates was carried out as previously described (3).

One phase of the study involved the incubation of erythrocytes in lightly buffered solutions of various hydrogen ion concentrations between pH 6.0 and 7.8. In these experiments phosphate buffers were used for the range pH 6.0 to 7.0 and glycylglycine buffers, for the range pH 7.0 to 7.8. The cell suspensions were prepared as follows: In a trial series a quantity of blood was introduced into each of a series of tubes containing a solution buffered respectively at pH 6.0, 6.20, 6.50, 6.75, 6.90, 7.10, and 7.4. Because of the buffering power of the blood itself the resultant pH, in most of the samples, deviated from that of the buffer solution. The pH in each case was adjusted to the original pH of the buffer by the addition of *N*/10 HCl or *N*/10 NaOH. After the volume of acid or alkali required in each case was noted, the specimens were discarded. Another series of the buffered solutions was then set up and the observed quantities of acid or alkali were added to the medium in the respective tubes. Finally, on addition of the blood, each specimen assumed the pH of the original buffer. This procedure obviated the necessity of adding the acid or alkali directly to the red-cell suspension.

Results

Metabolic Changes in Erythrocytes during Storage

The data represented in Fig. 1 indicate the metabolic behavior of the red cells during storage for 98 days in CD medium at 4° C with or without added adenosine or inosine, and in ACD medium with added inosine. The changes in the concentration of the organic phosphate intermediates in these samples were essentially as observed in a previous study (3). The utilization of the ribose moiety of the adenosine proceeded at a slow rate until about the 40th day and, as indicated in Fig. 2, varied inversely with that of glucose. As a rule, glucose utilization tends to be well maintained in the presence of adenosine whereas it soon becomes depressed in favor of ribose utilization in the presence of inosine. It is of interest that, in the experiment with adenosine, glucose was metabolized rapidly and, upon its depletion about the 50th day, the rate of utilization of ribose was increased. To obtain information concerning the influence of various conditions on the preferential utilization of glucose or ribose, the influence of the hydrogen ion concentration, of the temperature, and of the presence of ammonia was studied.

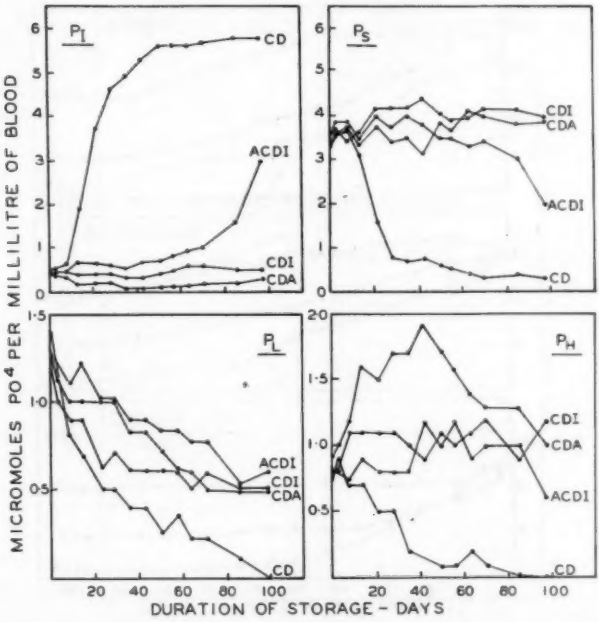


FIG. 1. Behavior of the phosphate fractions in human erythrocytes during storage at 4° C in CD and ACD media containing inosine or adenosine.

Legend: CD, isotonic citrate–dextrose medium (1.80 g sodium citrate and 1.47 g dextrose per 100 ml of solution, pH 7.4); ACD, acidified citrate–dextrose medium (0.44 g citric acid, 1.32 g sodium citrate, and 1.47 g dextrose per 100 ml, pH 5.5; when mixed with blood, the pH is about 7); CDA, citrate–dextrose medium containing 0.15 *M* adenosine (final concentration); CDI, citrate–dextrose medium with 0.15 *M* inosine; ACDI, acidified citrate–dextrose medium with 0.15 *M* inosine.

Influence of Hydrogen Ion Concentration

In a previous study (10) we had observed that, with variation in the hydrogen ion concentration of the medium, the hexokinase of the erythrocyte shows two optima of activity, namely at pH 6.0 and 7.8. In view of the rise in pH that occurs when adenosine is added to blood (3) it was of interest to ascertain whether the preferential utilization of glucose in the presence of adenosine might be related to the influence of the nucleoside in maintaining a more favorable hydrogen ion concentration for hexokinase activity. Ten blood samples, from a donation collected in the citrate–dextrose (CD) medium, were divided into pairs and each pair was adjusted to a definite pH within the range 6.0 to 7.8 by addition of the appropriate buffer. To one sample (designated CDI) of each pair inosine* was added. The samples then were incubated at 37° C for 18 hours, and the total quantity of glucose utilized was determined in the case of the CD and CDI samples, and the quantity

*The effect of inosine, when added to the specimens at the various hydrogen ion concentrations, is comparable with that of adenosine.

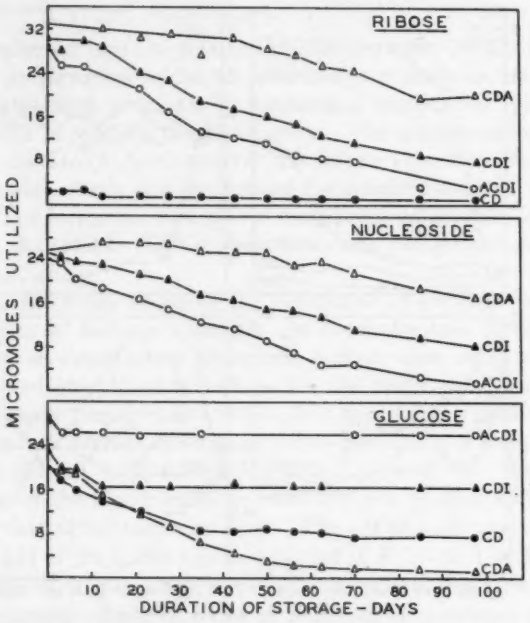


FIG. 2. Utilization of nucleosides, ribose, and glucose by erythrocytes preserved with adenosine and inosine at 4° C.
Legend: as with Fig. 1.

of ribose, in the CDI samples. The results of the experiment are represented in Fig. 3.

Despite the presence of the buffer, the pH of the specimens decreased during the incubation treatment. The magnitude of the shift was somewhat greater in the samples with the highest initial pH values because of the relatively greater hexokinase activity and the consequently increased rate of utilization of glucose and production of lactic acid. The points on the curves in the figure represent the means of the pH values at the beginning and at the end of the experiment. In general, the rate of glucose utilization in the various specimens corresponded to the hexokinase activity at the various hydrogen ion concentrations (10). The glucose utilization proved to be very sensitive to the increase in hydrogen ion concentration. As previously observed, the utilization of glucose in the sample with inosine was relatively small (9). In none of the samples, however, was the glucose utilization completely inhibited.

The system involved in the utilization of ribose, on the other hand, is relatively less sensitive to change in the concentration of H^+ ions. Thus the rate of utilization of ribose was not retarded until the pH of the specimens approached a low value (6.1). It is clear, therefore, that the preferential

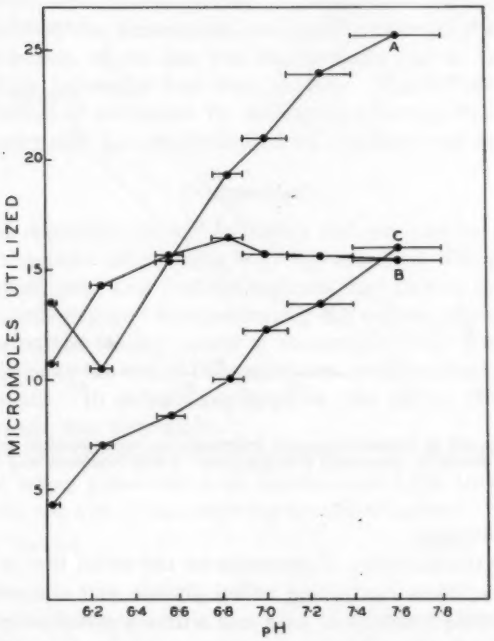


FIG. 3. Influence of hydrogen ion concentration on the utilization of glucose and ribose by preserved erythrocytes at 37° C. Each flask contained 1.5 ml whole blood, 0.25 *M* glucose, 0.15 *M* inosine, 0.2 *M* phosphate or glycylglycine buffer of appropriate pH (final concentrations). Total volume of preparation, 2.3 ml. The hydrogen ion concentration of the medium was adjusted to required pH with dilute NaOH or HCl before the blood was added. Duration of incubation: 18 hours. Temperature: 37° C.

Legend: A, glucose utilization in blood preserved in CD medium; B, ribose utilization in the CDI specimen; C, glucose utilization in blood preserved in CDI medium.

utilization of ribose or of glucose is, to a considerable extent, determined by the hydrogen ion concentration of the sample. The addition of adenosine to blood tends to maintain the pH at a comparatively high level because of the liberation of ammonia and thus favors a preferential and more prolonged utilization of glucose by the cells.

Influence of Temperature

One lot of blood specimens with added inosine and glucose was adjusted to pH 6.2, and another, to pH 7.5 by the addition of a suitable buffer. The samples then were maintained at various temperatures for 18 hours. The results of the experiment are indicated in Fig. 4.

The data indicate that at pH 7.5 the rate of utilization of both glucose and ribose was doubled with each 10° increase in the temperature. However, the utilization of glucose proved to be more sensitive than that of ribose to the combined decrease in the pH (to 6.2) and the temperature.

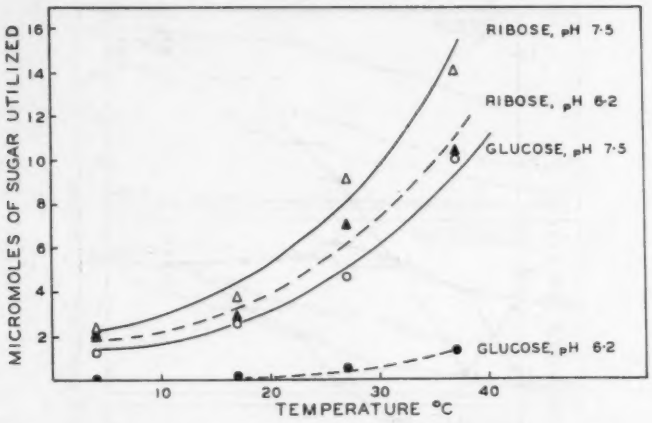


FIG. 4. Influence of temperature and hydrogen ion concentration on the utilization of glucose and ribose in preserved erythrocytes. Flask contents and conditions as in Fig. 3.

Influence of Ammonia

To ascertain the influence of ammonia on the utilization of the sugars one lot of blood specimens containing added glucose and adenosine, and others with added glucose and inosine with and without added ammonium chloride (0.15 *M* or 0.015 *M*), respectively, were maintained at 37° for 18 hours. The lower of the concentrations of NH_4Cl corresponds more closely to the concentration of ammonia liberated from the quantity of adenosine added to the one set of specimens.

The concentration of ammonium chloride added corresponds to that of the ammonia produced from the concentration of adenosine used in the one lot of specimens. The results indicate that in the presence of 0.15 *M* NH_4^+ ions the rate of utilization of glucose was very considerably increased while that of ribose was diminished. With the lower concentration of ammonium ions the degree of stimulation of the utilization of glucose was much diminished and was not always reproducible.

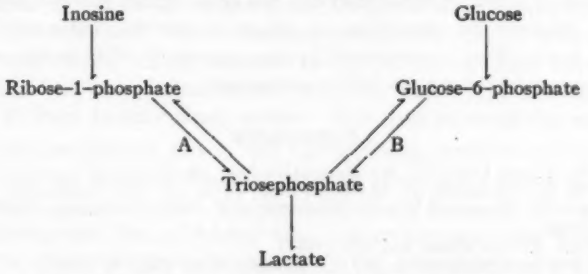
It is evident that the glucose utilization was favored in the presence of adenosine, while ribose utilization was dominant in the presence of inosine. In some instances the increased hydrogen ion concentration during storage of blood with inosine virtually arrested the utilization of glucose. The observed influence of adenosine and inosine on the metabolism of glucose and ribose in the red cells during preservation at 4° C is consistent with our former findings with blood at 37° C. The influence of ammonia liberated from adenosine is further substantiated by the observation that the addition of ammonium ions to the blood stimulated the utilization of glucose and depressed the utilization of ribose. It was of interest, therefore, to study the partition of ammonium ions between the cell and the serum at 4° and 37° C.

It is apparent that the ammonium ions readily entered the red cells and that the concentration of the ion was higher than that in the cells of the specimen to which adenosine had been added. The failure to reproduce completely the effect of adenosine by adding an ammonium salt along with inosine, therefore, could not be attributed to failure of the ammonium ions to enter the cells.

Discussion

In view of the relatively greater solubility and nontoxicity of inosine this nucleoside offers definite advantages over adenosine for the preservation of red cells for clinical use. Our findings indicate that inosine is as effective as adenosine in maintaining and in regenerating the organic phosphate esters in the red cells during the 98-day period of storage at 4° C. Furthermore, the degree of hemolysis at the end of the period was considerably less with inosine than with adenosine. In either case, however, the degree of the cell breakdown during storage was very slight.

The reason for the observed difference between the metabolic behavior of the erythrocyte when preserved with inosine and with adenosine may be made clearer with the aid of the following simplified scheme:



The accumulation of triosephosphate from pathway A would tend to inhibit utilization of glucose by way of pathway B. Likewise an accumulation of triosephosphate from reaction B would tend to inhibit the utilization of ribose. Whether pathway A or B will predominate depends on the pH and other conditions. In the citrate–dextrose medium, and especially in the presence of adenosine, the pH of the cells and the medium is higher because of the liberation of ammonia from the adenine moiety of the nucleoside. These conditions are favorable to the activity of hexokinase and the utilization of glucose.

In the CD medium with added inosine, on the other hand, the pH of the cells and the medium tends to fall more rapidly. The hexokinase activity and the glucose utilization become suppressed after the 13th day of storage, by which time the pH usually has decreased to about 7. Under these conditions the utilization of ribose is favored. The metabolic relation between ribose and glucose utilization is evident, furthermore, from the observation that although adenosine favors the utilization of glucose it does not prevent the utilization of ribose when the supply of glucose becomes exhausted.

In the ACD preservative medium with added inosine, on the other hand, the pH of the blood specimen at the outset is about 7.1. The glucose utilization therefore is inhibited from the beginning of storage and usually is brought to a standstill by the third day. Ribose then is preferentially utilized. In blood preserved in CD with inosine, the glucose utilization remains fairly active for about 13 days. There is evidence that adenosine stimulates the utilization of glucose by the red cells not only by maintaining the hydrogen ion concentration at a higher level but also through the influence of the ammonium ion itself. The presence of this ion apparently tends to retard the rate of ribose utilization and favor the utilization of glucose. To produce an increase in glucose utilization equal to that obtained with adenosine, it is necessary to add ammonium ions considerably in excess of the concentration that would accrue from the deamination of the concentration of adenosine ordinarily used in experimental blood preservation. The favorable effect of adenosine on the utilization of glucose by the cells is explainable, therefore, by the influence of this nucleoside on the pH of the sample and by the liberation of ammonia. The addition of potassium ions does not increase the glucose utilization.

Regardless of whether the preserved red cells utilize glucose preferentially under the influence of adenosine, or ribose under the influence of inosine, the energy metabolism, as reflected by the reserve of ATP in the cells during storage, appears to be equally well maintained.

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NOTE: The following abbreviations were used throughout this paper: CD, citrate–dextrose preservative medium; CDA, citrate–dextrose with adenosine; CDI, citrate–dextrose with inosine; ACD, acidified citrate–dextrose; ACDI, ACD with added inosine; P_I, inorganic phosphate; P_L, labile phosphate, mainly ATP, adenosine triphosphate; P_H, 'hydrolyzable' phosphate, mainly hexose-6-phosphate; P_S, stable organic phosphate, mainly DPG, 2,3-diphosphoglycerate.

NOTES

NOTE ON A RAPID TRANSLOCATION OF PHOTOSYNTHETICALLY ASSIMILATED C^{14} OUT OF THE PRIMARY LEAF OF THE YOUNG SOYBEAN PLANT[1]

C. D. Nelson, Harold J. Perkins, and Paul R. Gorham

Vernon and Aronoff (1) have shown that photosynthetically assimilated C^{14} is translocated downward in soybean plants chiefly in the form of sucrose and at a velocity of 84 cm per hour. The present work describes experiments which arose out of attempts to identify, by autoradiography, the tissue of the soybean plant in which such translocation occurs. In an attempt to locate the "front" of the radioactivity the time allowed for translocation was progressively shortened from 15 minutes to 30 seconds. Surprisingly, however, the true "front" could not be located since even in 30 seconds small amounts of C^{14} could be extracted from the stem immediately above the root. Whether this C^{14} was translocated as carbon dioxide or as an organic compound and whether it was localized in any particular tissue have not been determined. This note demonstrates that the velocity of this rapid translocation is at least twenty times greater than that reported for sugars.

Soybean plants (*Glycine max* L. var. Comet) were grown in coarse exploded mica (Vermiculite) irrigated from below with Hoagland's solution containing micronutrients, at a constant temperature of 21° C, under illumination of 2000 ft-c (tungsten), for a 16-hour day. Approximately one hour prior to the experiment the plants were moved to the laboratory where they were illuminated with 2000 ft-c of tungsten light measured at the level of the primary leaves. At zero time, approximately 50 μc of $C^{14}O_2$ (specific activity 8.86 mc per millimole CO_2) was injected from a hypodermic syringe into a small polyethylene bag which was sealed around one of the primary leaves of the plant. After 30 seconds, the fed primary leaf was severed from the plant at the base of the petiole and the remainder of the plant was sectioned as shown in the table. As each section of the plant was cut, it was frozen in liquid nitrogen. The time elapsed between severance of the fed leaf and freezing of the lowest section of stem did not exceed five seconds. Two methods of extraction of the plant material were used. The first (hot extraction) has been described previously (2); the second (cold extraction) involved (*a*) homogenization of the tissues at room temperature in a Ten Broeck tissue grinder with 80% aqueous ethanol, (*b*) centrifugation and concentration of the supernates and washings to dryness under reduced pressure at 30° C, and (*c*) re-solution of the residues in known volumes of 80% ethanol.

[1]Issued as N.R.C. No. 4924.

TABLE I

DISTRIBUTION OF C^{14} IN SOYBEAN PLANTS, 19 DAYS OLD, AFTER PHOTOSYNTHESIS IN $C^{14}O_2$ FOR 30 SECONDS BY A PRIMARY LEAF

Plant part	Plant 1 (hot extraction)		Plant 2 (cold extraction)	
	Length, cm	Total activity, d.p.m.†	Length, cm	Total activity, d.p.m.†
Stem tip, immature trifoliate leaf, and 2nd foliar node	—	459	—	546
Mature trifoliate leaf	—	1372	—	472
Stem between 1st and 2nd foliar nodes	6.0	36	4.0	15
			1.5	63
Treated primary leaf and 1st foliar node	—	10.9×10^6	—	10.9×10^6
Opposite primary leaf	—	4090	—	1306
Stem between 1st foliar node and root*	2.0	0	2.0	60
	2.0	31	2.0	14
	2.0	16	2.0	0
	2.0	0	2.0	7
	2.0	0	2.0	12
	2.0	82	2.0	10
	3.0‡	10	2.0	28
	3.0	35	2.0‡	19
	3.0	29	2.0	14
			2.0	33

*Sections of stems are arranged downward from the first foliar node to the root.
†All counts have been corrected for background, self-absorption, and counter efficiency. Zero means less than seven disintegrations per minute (d.p.m.).
‡Section of stem includes the cotyledonary node.

Aliquots of these solutions were plated on aluminum planchets and counted to within 3% standard error in a windowless methane gas flow counter operating in the proportional region.

The results of two typical experiments are shown in the table. Approximately 10% of the offered $C^{14}O_2$ was assimilated by the primary leaf during the 30-second exposure. There was translocation of C^{14} to the trifoliate leaves, to the opposite primary leaf, and to the lowest section of stem at least 18 cm from the first foliar node. If a time of 35 seconds and a distance of 18 cm were used for calculation, the minimum velocity of downward translocation of C^{14} was 1900 cm per hour. An even higher velocity (2400 cm per hour) was obtained if the length of the petiole of the treated leaf and the length of the lowest section of stem were included in the calculation. Further, the amount of C^{14} varied from one section of stem to another and in some sections no C^{14} could be detected. There was no appreciable difference between the two methods of extraction.

That the data were valid was determined as follows: (1) All planchets and glassware were checked for radioactive contamination. (2) No radioactivity could be detected in the stem of an untreated plant which was growing in the same pot as the treated plants during the experiment. (3) Doubling the aliquot on a planchet resulted in a doubling of the net count.

The experiment has been carried out with a total of 11 plants of various ages which were allowed to carry on photosynthesis as follows: four plants, 30 seconds; four plants, 1 minute; two plants, 2 minutes; and one plant, 5 minutes. In each plant the same general pattern of distribution of C^{14} was observed. The significance of this rapidly moving C^{14} in relation to the problem of translocation is being further investigated.

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CORRECTIONS

Volume 36

Page 808. In line 27, $K_e:K_e=Cl_e:Cl_e$ should read $K_c:K_e=Cl_e:Cl_c$.

Page 876. In line 12 up, C—O should read C=O; in line 9 up, C—C should read C=C.

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